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Redistribution of synaptic vesicles and their proteins in temperature-sensitive shibire^{ts1} mutant *Drosophila*

(cysteine string proteins/synaptobrevin/synaptotagmin)

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ABSTRACT **From an extract of *Drosophila melanogaster* head homogenates, a membrane fraction can be isolated that has the same sedimentation properties as vertebrate synaptic vesicles and contains *Drosophila* synaptotagmin. The fraction disappears from homogenates of temperature-sensitive (ts) mutant shibire^{ts1} (*shi*^{ts1}) flies paralyzed by exposure to nonpermissive temperatures, and reappears on return to permissive temperatures. Since reversible, temperature-dependent depletion of synaptic vesicles is known to occur in shibire^{ts1} flies, we conclude that the fraction we have identified contains synaptic vesicles. We have examined the fate of synaptic vesicle membrane proteins in shibire flies at nonpermissive temperatures and found that all of these vesicle antigens are transferred to rapidly sedimenting membranes and codistribute with a plasma membrane marker by both glycerol velocity and metrizamide density sedimentation and by confocal microscopy. Three criteria were used to establish that other neuron-specific antigens—neuronal synaptobrevin and cysteine-string proteins—are legitimate components of synaptic vesicles: cosedimentation with *Drosophila* synaptotagmin, immunoadsorption, and disappearance of these antigens from the vesicle fractions in paralyzed shibire flies.**

Synaptic vesicles contain high concentrations of neurotransmitter, which is released when exocytosis is triggered by calcium influx into the nerve terminal. To balance this loss of vesicles due to exocytosis, synaptic activity induces a rapid rate of endocytosis. Stimulation of endocytosis correlates with the dephosphorylation of a cytoplasmic nerve-terminal GTPase, dynamin, probably by the calcium-activated phosphatase, calcineurin (1, 2). Dynamin is known to play a major role in synaptic vesicle recycling from characterizations of the shibire mutation in *Drosophila* in which dynamin is defective. When flies bearing temperature-sensitive (ts) alleles of the shibire locus (*shi*) are exposed to high temperatures, they are paralyzed within a minute, and they recover rapidly when returned to permissive temperatures. Electron microscopy of the nervous system shows that paralysis correlates with depletion of synaptic vesicles from nerve terminals (3).

Because synaptic vesicles can be isolated, we can learn about the state of their proteins prior to docking and fusion. To identify the changes that synaptic vesicle proteins undergo as they fuse with the membrane and recycle back to form new vesicles, it would be advantageous to be able to freeze the terminal at different parts of the exo–endocytotic cycle. The temperature-sensitive alleles of shibire potentially provide a tool to halt the cycle after fusion but before endocytosis.

To take advantage of the only known ts mutation of vesicle recycling, the shibire mutation, we set out to develop biochemical assays for the synaptic vesicle content of the adult *Drosophila melanogaster* central nervous system. We identified synaptic vesicles from *Drosophila* using antibodies that recognize *Drosophila* synaptotagmin. We confirmed the identity of synaptic vesicles by the disappearance of synaptotagmin immunoreactivity from the synaptic vesicle fractions in shibire flies at nonpermissive temperatures. This property of shibire was then used as a criterion to establish that other *Drosophila* neuron-specific antigens, the neuronal synaptobrevin (n-Syb) and *Drosophila* cysteine-string proteins (Dcsps), are legitimate synaptic vesicle proteins. We have further shown that vesicle antigens move from vesicles to membrane that copurifies by subcellular fractionation techniques and colocalizes by confocal microscopy with a plasma membrane marker. Because the antigen-containing membranes have very different physical properties at permissive and nonpermissive temperatures, it is now possible to use subcellular fractionation of *Drosophila* membranes to look for exocytosis-induced changes in synaptic vesicle membrane proteins.

EXPERIMENTAL PROCEDURES

Generation of Antibodies. Rabbit polyclonal antisera NSYB1 and NSYB2 were generated against the 14 amino acids of the cytoplasmic domain (Met-Ala-Asp-Ala-Ala-Pro-Ala-Gly-Asp-Ala-Pro-Pro-Asn-Ala) and the intravesicular domain (Gln-Pro-Pro-Gln-Tyr-Gln-Tyr-Pro-Pro-Gln-Tyr-Met-Gln-Pro) of *Drosophila* n-Syb, respectively. To facilitate coupling of this peptide to the carrier protein (keyhole limpet hemocyanin), a cysteine residue was included at the N terminus. Antipeptide antisera were raised by Immuno-Dynamics (La Jolla, CA).

Antibody DSYT2 recognizing *Drosophila* synaptotagmin was a gift of Bellen and Littleton and coworkers (4). Monoclonal antibody to Dcsps, mAb49, was obtained from Buchner and Zinsmaier and coworkers (5). Rabbit IgG fraction to horseradish peroxidase (HRP) and secondary antibodies (HRP-conjugated affinity-purified goat anti-mouse and goat anti-rabbit IgG) were purchased from Cappel.

Depletion of Synaptic Vesicles in the shibire Fly. The experimental animals were the wild-type strain of *D. melanogaster* and the ts mutant strain carrying the shibire^{ts1} allele. Flies were cultured in standard sugar/agar-containing medium in bottles at 19°C. To deplete nerve terminals of synaptic vesicles, shibire flies were incubated at 32°C for 15 min, and the paralyzed flies were rapidly frozen in liquid nitrogen. To follow reformation of synaptic vesicles after depletion, shibire flies first depleted of synaptic vesicles were allowed to recover at 19°C for 30 min prior to freezing.

Subcellular Fractionation. Flies (10–15 g) frozen in liquid nitrogen were decapitated, and heads were collected with a sieve. Frozen heads were ground in a mortar and pestle. All subsequent steps were done at 4°C. The resulting powder was resuspended in 1 ml of buffer A (150 mM NaCl/10 mM Hepes,

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Abbreviations: ts, temperature sensitive; csp, cysteine-string protein; Dcsps, *Drosophila* csps; HRP, horseradish peroxidase; Syb, synaptobrevin; n-Syb, neuronal Syb.
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pH 7.4/1 mM EGTA/0.1 mM $MgCl_2$ without inhibitors) and homogenized by 20 strokes in a glass–glass homogenizer. The postnuclear supernatant (10 min at 1000 × g) was layered on 5–25% glycerol gradient in buffer A over a 50% sucrose pad (6). Gradients were centrifuged for 1 h at 48,000 rpm in a SW 55 Ti rotor (Beckman). For equilibrium density sedimentation, the supernatant was layered on 20–55% metrizamide gradients in buffer A and centrifuged to equilibrium buoyant density for 20 h at 55,000 rpm. Gradient fractions were analyzed by Western blotting using the enhanced chemiluminescence (ECL) system (Amersham).

Immunoadsorption. Postnuclear supernatant of *Drosophila* heads was subjected to two additional centrifugations for 15 min each at 14,000 × g to pellet large membranes. The supernatant was divided in three 200-μl aliquots. Triton X-100 was added to one aliquot to 1% final concentration. Aliquots were incubated with rotation for 10 min. Thirty microliters of mAb49 was added, and incubation was continued for 4 h. Ten microliters of protein G-Sepharose 4 fast flow (Pharmacia LKB) was added and incubated 30 min with rotation. Sepharose beads were collected by centrifugation for 4 min at 1000 × g. Pellets were washed three times with 0.5 ml of buffer A or buffer A/1% Triton X-100 and analyzed by Western blotting.

Confocal Microscopy. Confocal microscopy was carried out as described in Ramaswami et al. (7).

RESULTS

Identification of *Drosophila* Synaptic Vesicles and Their Sedimentation Properties. *Drosophila* synaptotagmin is expected to be a good marker for synaptic vesicles in adult *Drosophila*. The protein is evolutionarily conserved between *Drosophila* and mammals. Antibody to the *Drosophila* protein (DSYT2) specifically stain nerve terminals, and mutations in the synaptotagmin gene affect transmitter release (8). In addition the protein is present in slowly sedimenting fractions where small vesicles are recovered (4). Therefore, we used the DSYT2 antiserum to characterize, by velocity sedimentation of *Drosophila* head extracts, the membranes that contain this protein.

By subcellular fractionation of wild-type *Drosophila* homogenate, synaptotagmin antigenicity was detected in two distinct membrane populations: rapidly sedimenting membranes on the pad at the bottom of the gradient (fractions 2 and 3 in Fig. 1*A*), which presumably contain endosomes and plasma membrane, and a slowly sedimenting homogeneous fraction (fractions 9–12 in Fig. 1*A*) with the same sedimentation rate as rat brain synaptic vesicles (6). This result is consistent with morphological studies showing that *Drosophila* synaptic vesicles have a similar diameter (40–50 nm) to those in rat (9).

It has been reported that DSYT2 antibody recognizes full-length synaptotagmin (69 kDa) and a protein of 55 kDa that is probably a breakdown product of synaptotagmin (4). In all of our preparations, detection of both forms of the protein was independent of the presence or absence of proteolytic inhibitors. Moreover, antiserum generated in our laboratory to *Drosophila* synaptotagmin also recognized both forms, suggesting that the 55-kDa band is not an unrelated cross-reacting protein.

We used *Drosophila* protein Rop (a sec1p-related protein) as a marker for proteins that are not targeted to synaptic vesicles. Rop is expressed in the nervous system and other tissues that are actively engaged in secretion and is required for vesicle transport (10). Crude subcellular fractionation and immunohistochemistry demonstrated that Rop is both cytoplasmic as well as peripherally associated with large membranes (10). In our velocity sedimentation experiments, the membrane-bound Rop was exclusively associated with the large membranes migrating at the bottom of the glycerol

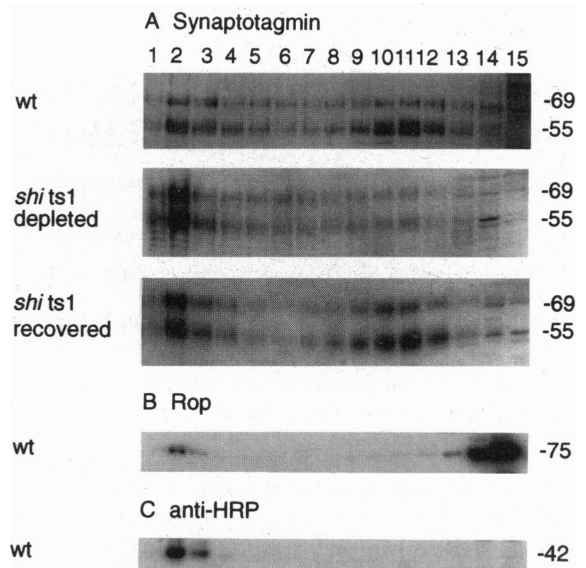

FIG. 1. Depletion of synaptic vesicles in shibire flies analyzed by glycerol velocity sedimentation. Heads from wild-type (wt) *Drosophila*, $shibire^{ts1}$ mutant *Drosophila* at nonpermissive temperatures (shi^{ts1} depleted) and $shibire^{ts1}$ *Drosophila* first paralyzed and then recovered at 19°C (shi^{ts1} recovered) were collected and homogenized as described in text. Postnuclear supernatants (10 min at 1000 × g) containing equal amounts of total protein were layered on 5–25% glycerol gradients in buffer A over a 50% sucrose pad. Gradients were centrifuged for 1 h at 48,000 rpm in a SW 55 Ti rotor (Beckman). Fractions were collected from the bottom, numbered 1–15, and analyzed by Western blot analysis for the presence of synaptotagmin (*A*), Rop protein (*B*), and the 42-kDa plasma membrane anti-HRP antigen (Na^+/K^+-ATPase β subunit) (*C*). Bands were visualized by the enhanced chemiluminescence system. Note that the immunoreactivity is absent from the synaptic vesicle fractions 9–12 and is transferred to the plasma membrane-containing fractions 2 and 3 after shibire flies were incubated at a nonpermissive temperature (shi^{ts1} depleted). The immunoreactivity reappears again in the synaptic vesicle fractions when shibire flies first depleted of synaptic vesicles were allowed to recover at 19°C (shi^{ts1} recovered). Numbers on the right indicate molecular mass in kDa. The 55-kDa band in *A* is probably a breakdown product of synaptotagmin (4).

gradient (fractions 2 and 3 in Fig. 1*B*) and was excluded from the synaptic vesicle-containing fractions. The cytosolic pool of this protein sedimented at the top of the gradient (fractions 14 and 15, Fig. 1*B*).

To identify the position of plasma membrane in these velocity gradients, we used an anti-HRP antibody that has been shown to recognize a neural-specific cell surface carbohydrate epitope in *Drosophila* and other insects (11, 12). In a Western blot analysis of *Drosophila* homogenates, two major bands (42 and 80 kDa) can be seen (13). The 42-kDa protein has recently been identified as a homologue of the membrane-bound β subunit of the Na^+/K^+-ATPase and was found to be present on the plasma membrane (P. Salvaterra, personal communication). We used, therefore, the 42-kDa protein as a marker for plasma membrane. The immunoreactivity was consistently detected in the rapidly sedimenting fractions of the glycerol velocity gradient (fractions 2 and 3 in Fig. 1*C*).

Synaptic Vesicles Are Depleted in the $shibire^{ts1}$ Mutant at Nonpermissive Temperatures. To study the fate of synaptic vesicle membrane after exocytosis, we took advantage of the unusual properties of the $shibire^{ts1}$ mutation. In $shibire^{ts1}$ flies, elevated temperatures (above 27°C) cause rapid and reversible paralysis, and depletion of synaptic vesicles as has been demonstrated by morphological studies of individual nerve terminals (3, 9, 14, 15). This depletion is associated with a ts block in the endocytotic retrieval of synaptic vesicle membrane (7, 9). We attempted to detect such depletion by subcellular frac-

tionation. Postnuclear supernatant prepared from shibire^{ts1} flies incubated for 15 min at 32°C was centrifuged on a glycerol velocity gradient. Antibodies to a synaptic vesicle marker synaptotagmin showed a significant decrease of immunoreactivity in the synaptic vesicle-containing fractions 9–12 (*shi*^{ts1} depleted, Fig. 1*A Middle*). When an aliquot of shibire flies first depleted of synaptic vesicles was allowed to recover at 19°C for 30 min prior to homogenization, immunoreactivity was detected again in the synaptic vesicle-containing fractions, consistent with a reversible block in vesicle biogenesis (*shi*^{ts1} recovered, Fig. 1*A Bottom*).

Therefore, glycerol velocity sedimentation appears to allow the detection of synaptic vesicles. We found that the *Drosophila* synaptic vesicles (*i*) have the same sedimentation characteristics as rat synaptic vesicles. (*ii*) contain the well-established synaptic vesicle protein synaptotagmin, and (*iii*) dramatically decrease in shibire terminals at nonpermissive temperatures and reappear after the temperature has been lowered.

***Drosophila* n-Syb and csps Are Targeted to Synaptic Vesicles.** A second conserved protein of vertebrate synaptic vesicles is Syb. The expression profile and tissue distribution of *Drosophila* n-Syb match that of a synaptic vesicle protein (16). To identify the subcellular localization of n-Syb in *Drosophila*, we generated antisera to peptides from its intravesicular and cytoplasmic domains. Antibodies to both domains of n-Syb recognized a single band of ≈23 kDa on a Western blot of a postnuclear supernatant from *Drosophila* heads (Fig. 2*A*). The migration of this protein reflects the higher molecular weight of n-Syb compared with the other isoform (Syb, 20 kDa) in *Drosophila* that is expressed only in low levels in the nervous system and is most abundant in the gut (17). Since the peptides used for immunizations were not conserved between n-Syb and Syb and since we failed to detect a 20-kDa band, we conclude that this antibody is specific for n-Syb and does not crossreact with the nonneuronal Syb. The distribution of n-Syb after velocity sedimentation of head extracts (Fig. 2*A*) supports its targeting to synaptic vesicles. This is confirmed by its redistribution in shibire flies at nonpermissive temperatures and after recovery at 19°C. Thus, n-Syb is a synaptic vesicle protein with a subcellular distribution and traffic indistinguishable from that of synaptotagmin.

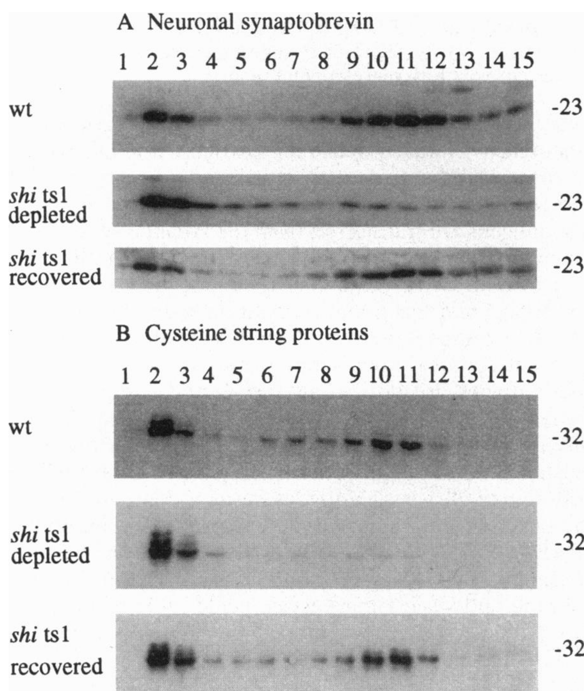

FIG. 2. Localization and redistribution of n-Syb and csps. Postnuclear supernatants and glycerol velocity sedimentations were identical to those in Fig. 1. Localization of n-Syb (*A*) and Dcsps (*B*) in wild-type (wt) flies and their redistribution in shibire^{ts1} mutant *Drosophila* at nonpermissive temperatures (*shi*^{ts1} depleted) and after decreasing the temperature back to 19°C (*shi*^{ts1} recovered) are identical to those of synaptotagmin, indicating that they are synaptic vesicle proteins. Numbers on the right indicate molecular masses in kDa.

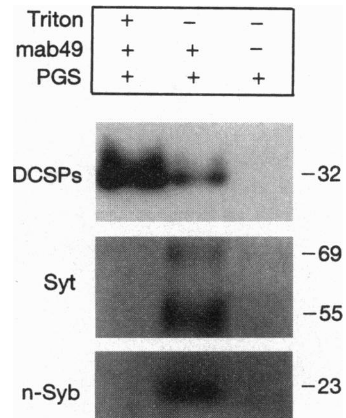

FIG. 3. Immunoadsorption of *Drosophila* synaptic vesicles. Aliquots of postnuclear supernatant of wild-type *Drosophila* heads (containing small vesicles but devoid of large membranes) were incubated with mAb49 directed against Dcsps with and without 1% Triton X-100, followed by protein G-Sepharose 4 fast flow (PGS). Adsorbed proteins were separated by SDS/PAGE and analyzed by Western blot analysis for the presence of Dcsps, synaptotagmin (Syt), and n-Syb. In one aliquot antibody was omitted to determine the background caused by the beads alone. Numbers on the right indicate molecular masses in kDa.

csps were initially identified in *Drosophila* as synapse-specific antigens that contain a unique region rich in cysteine residues (5). Deletion of the Dcsp gene interferes with synaptic transmission causing paralysis and early death (18). In *Torpedo*, csp is predominantly a synaptic-vesicle antigen (19). Using velocity sedimentation, we investigated the subcellular localization of Dcsps and their redistribution after massive exocytosis in shibire^{ts1} flies. Postnuclear supernatants of wild-type and shibire^{ts1} heads at nonpermissive temperatures and after recovery at permissive temperatures were fractionated on glycerol velocity gradients as described above. We found that the Dcsp immunoreactivity over these gradients parallels that of synaptotagmin and n-Syb (Fig. 2*B*). Moreover, antibody to Dcsps coimmunoisolated n-Syb and synaptotagmin from a high-speed supernatant (containing synaptic vesicles but devoid of plasma membrane) but immunoisolated only Dcsps after solubilization with 1% Triton X-100 (Fig. 3). Thus, we can conclude that although Dcsps lack transmembrane domain, they are present on synaptic vesicles and recycle together with other synaptic vesicle markers between synaptic vesicles and plasma membrane.

Synaptic Vesicle Markers Are Transferred from the Synaptic Vesicle Pool to the Plasma Membrane-Containing Fractions. The vesicle-recycling hypothesis proposes that during exocytosis synaptic vesicle membrane fuses with the plasma membrane and is subsequently retrieved to form new vesicles. This hypothesis would predict that after vesicle depletion in synapses of shibire^{ts1} flies, a substantial increase in the nerve terminal plasma membrane area would be observed because of the addition of synaptic vesicle membrane.

The depletion of all three synaptic vesicle antigens from synaptic vesicle-containing fractions 9–12 was accompanied by an increase of immunoreactivity in the rapidly sedimenting membranes that cosedimented with the plasma membrane marker (Figs. 1*A* and 2). To determine if the rapidly sedimenting vesicle antigens were associated with plasma membranes or some other organelle, we utilized a two-step isola-

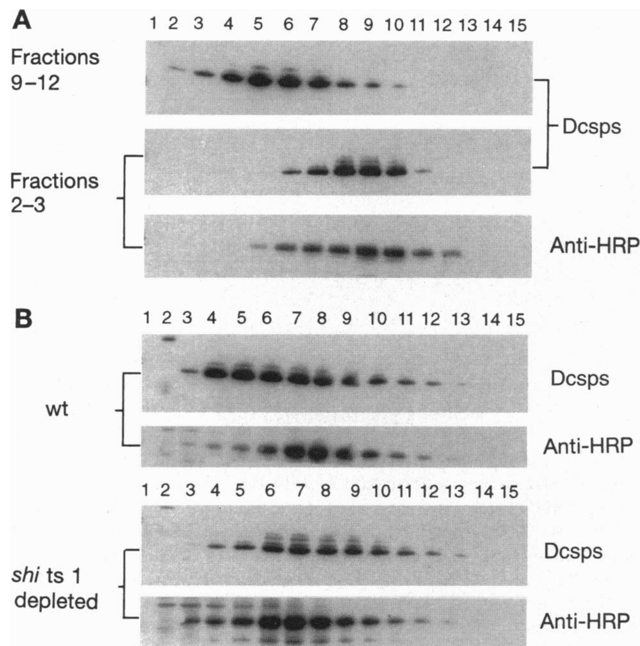

FIG. 4. Transfer of synaptic vesicle markers to fractions cosedimenting with the plasma membrane marker in metrizamide density sedimentation. (*A*) A postnuclear supernatant of *Drosophila* heads (10 min at 1000 × *g*) was fractionated by glycerol velocity sedimentation, and fractions containing the large membranes (2–3) or synaptic vesicles (9–12) were pooled. Both pools were further fractionated on 20–55% metrizamide gradients for 20 h at 55,000 rpm in a SW 55 Ti rotor (Beckman). Fractions were numbered from the bottom and assayed by Western blot analysis for the presence of Dcsps and the 42-kDa plasma membrane marker (anti-HRP). On these gradients synaptic vesicle-containing fractions are denser than large membrane fractions. Immunoreactive Dcsps in the large membrane fractions cosediment with the plasma membrane marker. (*B*) A postnuclear supernatant from shibire flies depleted of synaptic vesicles by incubation at 32°C for 15 min (*shi*^{ts1} depleted) and from wild-type (wt) flies was directly fractionated on a 20–55% metrizamide gradient and analyzed as described in *A*. The Dcsp immunoreactivity in wt flies is spread over a large number of fractions. Note that of these only the less dense ones contain the plasma membrane marker. In shibire flies depleted of synaptic vesicles, the Dcsp immunoreactivity is shifted to the less dense fractions, so that all csp-containing fractions cosediment with the plasma membrane marker.

tion. To separate synaptic-vesicle membranes from the rapidly sedimenting membranes, we first fractionated the wild-type *Drosophila* head postnuclear supernatant by glycerol velocity sedimentation and pooled rapidly sedimenting fractions (fractions 2 and 3) and synaptic vesicles (fractions 9–12). Both pools were further fractionated by metrizamide density sedimentation (Fig. 4*A*). The two pools had different densities, the synaptic vesicle membranes sedimenting to a higher density than the rapidly sedimenting ones. Membranes carrying the plasma membrane marker had a density indistinguishable from the rapidly sedimenting membranes containing vesicle antigens. Thus, a significant fraction of synaptic vesicle antigens is associated with another membrane, presumably the plasma membrane, in extracts of wild-type heads.

Since synaptic vesicles and plasma membrane have different densities in metrizamide density gradients we could examine the fate of the synaptic vesicle antigens in shibire terminals at nonpermissive temperatures. Postnuclear supernatants from wild-type and shibire flies at nonpermissive temperatures were analyzed by density equilibrium sedimentation (Fig. 4*B*). Since the samples contained both synaptic vesicles and the rapidly sedimenting membranes, in wild-type flies immunoreactivity was spread over a large number of fractions, of which only the less dense ones cosedimented with the plasma membrane

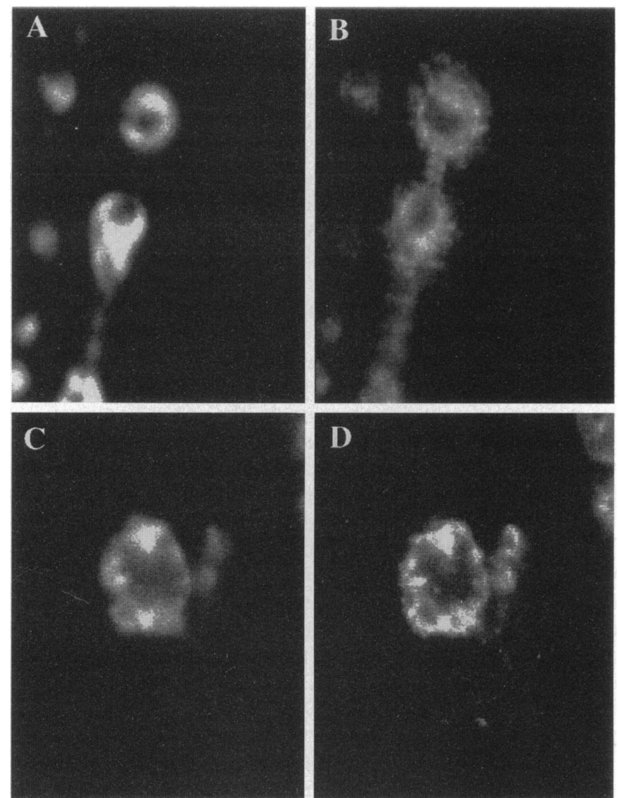

FIG. 5. Fine localization and redistribution of synaptic vesicle antigens in wild-type and shibire presynaptic terminals by confocal microscopy. (*A* and *B*) Wild-type terminals stimulated at 34°C and double-stained with anti-Dcsp (*A*) and anti-HRP (*B*). Dcsp immunoreactivity is localized to compact zones within a ring of presynaptic plasma membrane. (*C* and *D*) *Shi*^{ts1} terminals stimulated at 34°C and double-stained with anti-Dcsp (*C*) and anti-HRP (*D*). The synaptic vesicle antigen is now found in a ring-shaped structure which colocalizes extensively with plasma membrane.

marker. In shibire flies depleted of synaptic vesicles, Dcsp immunoreactivity was shifted to the less dense fractions so that all Dcsp-containing fractions now cosedimented with the plasma membrane marker. Thus, in depleted shibire terminals, synaptic vesicle antigens are transferred from the synaptic vesicle pool to the membranes that cofractionate with the plasma membrane marker by both velocity and density sedimentation.

The subcellular fractionation data on redistribution of synaptic vesicle antigens were confirmed by confocal microscopy. Our recent confocal microscopy results showed that synaptic vesicle antigens are not evenly distributed within the nerve terminals but accumulate within clusters. Stimulation of shibire nerve terminals at nonpermissive temperatures caused a redistribution of the antigens to ring-like structures (7). The distribution of the vesicle antigens was now compared with that of the plasma membrane marker, recognized by antibodies to HRP (Fig. 5). The clusters of vesicles lie within the plasma membrane in wild-type flies (Fig. 5 *A* and *B*), but the plasma membrane- and vesicle markers coincide after stimulation of shibire flies at high temperatures (Fig. 5 *C* and *D*), consistent with our subcellular fractionation data.

DISCUSSION

We have established velocity gradients to fractionate and enrich synaptic vesicles containing synaptotagmin. These vesicles have a unique sedimentation velocity on velocity gradients and are clearly separated from soluble and plasma membrane proteins. By this criterion, the *Drosophila* protein n-Syb and Dcsps are legitimate components of synaptic vesicles.

Although velocity sedimentation is often used to define synaptic vesicles in preparations from mammalian cells, other vesicles of small but homogeneous dimensions can be found (20). Because shibire^{ts} mutants with temperature-sensitive block in synaptic vesicle recycling are available, we are able to use a novel criterion to identify synaptic vesicles in *Drosophila*. Most central and peripheral synapses of shibire^{ts1} flies are depleted of synaptic vesicles by a 10-min incubation at 32°C (9); under these conditions, synaptotagmin, n-Syb, and Dcsps are dramatically absent from our synaptic vesicle peak and appear instead in fractions of rapidly sedimenting membranes. By combining this technique with immunoadsorption methods (Fig. 3), it is now possible to determine whether a given *Drosophila* presynaptic protein is a component of synaptic vesicles. Furthermore, it may be possible to purify proteins enriched in our vesicle peak that meet our criteria for synaptic vesicle proteins. Such an approach should help to identify the *Drosophila* homologues of mammalian synaptic vesicle proteins such as synaptophysin and SV2, and perhaps new proteins that have to date eluded investigators.

The fraction of synaptic vesicle antigens in larger membranes with the density of plasma membranes increases greatly in shibire^{ts1} flies at nonpermissive temperatures. Although the vesicle hypothesis for transmitter release and current models for shibire function imply that synaptic vesicle protein must be trapped on plasma membrane in shibire^{ts} mutants, this prediction was not supported by the electron microscopic studies on the cervical and coxal neuromuscular junctions by Koenig and Ikeda (15). Their quantitative measurements of vesicle and nerve terminal surface area for shibire flies showed no significant increase in the plasma membrane surface after vesicle depletion, which led these authors to propose an alternative scheme for synaptic vesicle recycling. Our subcellular fractionation data, suggesting an accumulation of vesicle antigens on the plasma membrane, are consistent with immunofluorescence studies on the shibire^{ts1} mutants (ref. 7 and Fig. 5) and support transfer of vesicle antigens to the plasma membrane.

Although electron microscopy revealed that terminals can become depleted of synaptic vesicles in shibire^{ts1} at nonpermissive temperatures, no estimates were available of how many synapses were affected. Even a failure of a few brain synapses could lead to paralysis if it results in the cessation of activity by other cells in the brain. We find that synaptic vesicles are almost completely absent from shibire^{ts1} *Drosophila* heads when shibire^{ts1} flies are incubated at nonpermissive temperature. Since depletion is activity-dependent, this means that there is sufficient synaptic activity in the brain to deplete almost all vesicles in almost all synapses of the central nervous system in 15 min at 32°C. One possibility is that the reserve vesicle pool of the brain can maintain synaptic activity for 15 min or less when it is not replenished. Alternatively, the temperature shift could induce massive abnormal synaptic activity that depletes most synapses, excitatory or inhibitory (21). Since the rate of loss of vesicles in a shibire^{ts} fly is a measure of synaptic activity, the system we present here can be used to examine further mutations for an effect on exocytosis. Similarly the kinetics of vesicle reappearance on return to permissive conditions gives a quantitative measure of the efficiency of synaptic vesicle biogenesis.

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